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DEGRADATION OF LYCORINE BY *PSEUDOMONAS* SPECIES STRAIN ITM 311

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ABSTRACT.—A lycorine-degrading bacterium was isolated from the rhizosphere of *Sternbergia lutea* Ker Gawl plants. The organism, *Pseudomonas* sp., strain ITM 311, was grown on a minimal medium with lycorine. Within 24-48 h, the alkaloid was transformed into three phenanthridinium derivatives (**2**, **3**, and **4**), two of which showed marked antibiotic activity. The evidence obtained demonstrates that the transformation consisted, essentially, in the oxidation of lycorine with an aromatization of the C ring and the conversion of the methylene group $H_2C(7)$-N into an azomethyne group.

Lycorine (**1**) (1) is an alkaloid known to occur in several plant species of the family Amaryllidaceae, including *Sternbergia lutea* Ker Gawl, *Clivia Miniata* Regel, *Zephyranthes candida* Herb., and many others (2).

Lycorine has a number of interesting biological activities (3-7) mediated by its action on the ascorbate system (8). The ability of lycorine to inhibit the ascorbic acid (AA) biosynthesis has made this substance a valuable tool for studying the AA-dependent reactions in those microrganisms that synthesize AA.

Growth of *Escherichia coli* and that of many yeasts is not inhibited by lycorine (9), and it has been suggested that the insensitivity of these microrganisms is due to their incapacity to synthetize AA or to the capacity to degrade lycorine. However, little is known about the biodegradation of lycorine and its biocidal activity.

In this report, we describe the isolation and identification of the metabolic products of a lycorine-degrading bacterium. Furthermore, the relationship between the chemical structure of these products and their antibiotic activity is discussed, also in comparison to that of some derivatives prepared by synthesis from **1**.

RESULTS AND DISCUSSION

The metabolic transformations described in this study occurred under culture conditions in which a *Pseudomonas* sp. can actively degrade lycorine. After the strain ITM 311 was incubated with lycorine for 48 h, nearly 100% of the substrate was degraded, and three products (**2**, **3**, and **4**) were recovered. Time course experiments indicated that lycorine was first transformed into products **2** and **4**.

The total yield of products **2** and **3** was about 7% of the starting material. In any case, large amounts of **2** and especially of **4** were found in the crude extracts of the bacterial masses grown in minimal medium liquid (MML). This indicates that **2** and **4** probably accumulated in the bacterial cells.

The transformation of lycorine was due to the bacterial activity. In fact, when lycorine (1%) was incubated with 2 ml of enzyme preparation (10 mg/ml of protein), all three products were detected after 24 h of incubation at 25°. On the contrary, any degradation of lycorine was observed when the alkaloid was incubated with strain ITM

311 cells treated at 80° for 15 min. Controls also indicated that lycorine itself was not transformed into any compounds examined in this work.

Of the three products, the main one was **2**, which was obtained as brilliant orange needles. Its ^{1}H-nmr spectrum (Table 1) was consistent with a lycorine-type structure having some structural modifications on the B and C rings with respect to **1**. An accurate analysis made in comparison with the ^{1}H-nmr spectrum of **1** (10) showed the absence of the signals attributed to H-2, H-11b, and H-11c and the downfield shift of the signals due to H-1 and H-3 ($\Delta\delta$=3.02 and 1.56 ppm, respectively) which appeared as a doublet at 7.60 δ and a double triplet at 7.33 δ, respectively. Furthermore, the AB system due to H_2C-7 in the spectrum of **1** disappeared, producing a singlet at 9.32 ppm.

TABLE 1. ^{1}H nmr of Compounds **2, 3, 5, 6, 7** (Chemical Shifts in ppm (δ) from TMS)

	2[a]	3[a]	5[b]	6[b]	7[c]
H-1[d]	7.60 d	8.52 d	7.28 d	7.08 d	—
H-2[d]	—	7.95 dd	6.75 dd	6.52 dd	7.32 d
H-3[d]	7.33 dt	7.86 dt	7.00 dt	6.62 dt	7.67 dt
2H-4[d]	3.73 td	3.84 td	3.02 td	2.76 td	3.71 td
2H-5[d]	5.22 t	5.30 t	3.32 t	3.82 t	5.24 t
H-7	9.32 s	9.61 s	4.06 s (2H)	—	9.55 s
H-8[e]	7.66 s	7.78 s	6.63 s	6.58 s	7.77 s
H-11[e]	7.96 s	8.27 s	7.16 s	6.88 s	9.03 s
2H-12	6.36 s	6.39 s	5.97 s	5.46 s	6.35 s

J (Hz), **2**: 1,3=3,4=1.8; 4,5=7.0; **3, 5** and **6**: 1,2=2,3=8.1; 3,4=1.5; 4,5=7.4; **7**: 2,3=8.0; 3,4=1.5; 4,5=7.3.

[a]Solvent used CD_3OD-CD_3COOD, 3:1.

[b]Solvent used $CDCl_3$.

[c]Solvent used CD_3OD.

[d]Assigned by evidence obtained from ^{1}H resolution enhanced spectrum and by ^{1}H nmdr.

[e]Attributed by comparison with the data reported for lycorine (10).

These results suggest structure **2** for the product isolated in the betaine form, which may be described by two resonance forms. As compared to that of **1**, the structure proposed provided the aromatization of the C ring and the oxidation of the H_2C-7. This was supported by the mass spectrum, which provided a parent ion peak at 265 *m/z*. The uv spectrum was characteristic of a phenanthridinium salt with a maximum absorbance at 258 and 361 nm, while a bathochromic shift was observed when the spectrum was recorded using 0.1 N NaOH as a solvent. The presence in **2** of an additional benzene ring was confirmed by ^{13}C-nmr data (Table 2). As compared to those of **1** (10), the proton noise decoupled and the single frequency off-resonance decoupled spectra essentially showed the presence of the carbon resonances due to another tetrasubstituted benzene ring and to an azomethyne group. The signals attributed in the spectrum of **1** to the carbons of the cyclohexene ring and to one of the methylene groups attached to the nitrogen atom were absent. Finally, the betaine **2** was identical (mp, ^{1}H nmr, ms, uv, and Rf in two tlc systems) with the compound prepared by the oxidation of lycorine according to the reported procedure (11).

The second metabolite (**3**) had a blue fluorescence that was revealed by exposing the plates used for its tlc analysis to uv radiation; this compound was purified from the residue left by the evaporation of the ethanolic mother liquors of the crystallization of **2**. To isolate **3**, it was necessary to repeat three times the chromatography on Sephadex LH-20 column.

The comparison of its ^{1}H-nmr spectrum (Table 1) with that of the anhydrolycorine (**5**) (Table 1) indicated the absence of the signals of the AB system due to H_2C-7 as the

TABLE 2. Carbon Shifts of Compound **2** and **3** (CD_3OD-CD_3COOD, 3:1) Chemical Shifts are in ppm (δ) from TMS

	2		**3**	
C-1[a]	104.12	d	104.53	d
C-2	162.59	s	111.68	d
C-3[a]	118.19	d	121.72	d
C-3a	139.82	s	138.12	s
C-4[a]	28.35	t	30.29	t
C-5	57.05	t	56.44	t
C-7[a]	141.97	d	139.80	d
C-7a[b]	126.22	s	129.40	s
C-8	108.22	d	108.74	d
C-9[b]	157.26	s	158.90	s
C-10[b]	151.84	s	155.32	s
C-11	102.02	d	102.41	d
C-11a[b]	124.09	s	127.37	s
C-11b	132.02	s	130.76	s
C-11c[a]	132.92	s	132.76	s
C-12	105.48	t	105.71	t

[a]Assignments supported by evidence obtained from sfsd spectra.

[b]Attributed by comparison with the resonance frequencies of the carbons of the corresponding ring of lycorine (10).

only difference, while a singlet due to an azomethyne group appeared at 9.61 δ. The mass spectrum gave a molecular ion peak at 250 *m/z*, and the uv spectrum showed the maximum absorbance at 258, 270, and 280 nm.

These data suggested that this metabolite has the structure of an anhydrolycorinium salt (**3**). The ^{13}C-nmr spectrum (Table 2) was in agreement with the structure proposed. The quaternary ammonium salt is chloride because when its aqueous neutral solution was treated with Ag_2O, the precipitation of AgCl and the formation of the corresponding quaternary ammonium hydroxide, raising alkalinization of the solution, were observed. Moreover, the anhydrolycorinium chloride **3** was very similar (mp, ^{1}H nmr, ms, uv, and Rf in two different tlc systems) to the compound prepared by the reaction of lycorine with $POCl_3$ (12).

The third product was probably the dihydroderivative of **2** (4), which had a sky-blue fluorescence when its tlc plates were exposed to uv radiation. In fact, by tlc analysis (SiO_2 plates, eluent *n*-BuOH-HOAc-H_2O, 60:15:25 and reversed phase C-18 plates, eluent H_2O-acetonitrile 3:2), this product was identical to the compound obtained, in accordance with the reported procedure (11), by catalytic reduction of **2**. All attempts to isolate **4** failed because of its well-known instability (11 and references cited therein). In fact, **4** easily reverts to **2**.

All the data presented show that the transformation of lycorine into the products **2** and **3** consists, essentially, in the oxidation of lycorine, and in particular the aromatization of the C ring and the conversion of the methylene group $H_2C(7)$-N into an azomethyne group was observed.

The pure products **2** and **3** were able to inhibit the growth of *Corynebacterium fascians* (Table 3) as well as the growth of other Gram-positive and Gram-negative bacteria but not that of strain ITM 311 (data not shown).

Lycorine, the anhydrolycorine (**5**) (13), the anhydrolycorine lactam (**6**) (13), the derivative **7** (11), which is a structural isomer of the **2** hydrochloride (**8**), and narciclasine (**9**) (14), another alkaloid-related substance extracted from plants of the Amaryl-

TABLE 3. Sensitivity of *Corynebacterium fascians*[a] (strain NCPPB[a] 2551) to Lycorine and Some Related Substances

Compound	Diameter of inhibition zone in mm[b]			
	Molarity of solutions used to impregnate paper discs for bioassay			
	0.01	0.001	0.0005	0.0001
1	0.0[c]	0.0	0.0	0.0
2	15.0	14.0	0.0	0.0
3	30.0	24.0	16.0	10.0
5	25.0	25.0	15.0	0.0
6	25.0	12.0	9.0	0.0
7	10.0	7.0	0.0	0.0
8	0.0	0.0	0.0	0.0
9	0.0	0.0	0.0	0.0

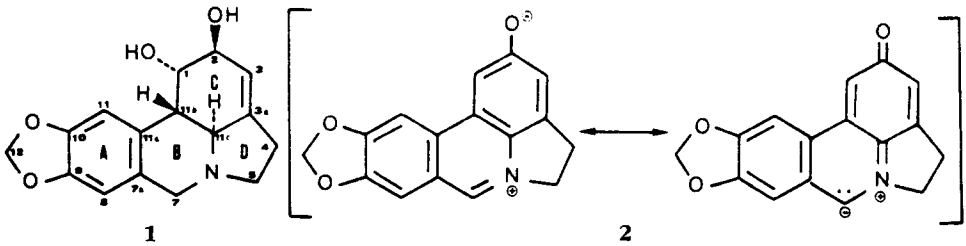
[a]National Collection of Plant Pathogenic Bacteria, Harpenden, England.
[b]Paper discs were impregnated with 20 µl of the different compounds in MeOH.
[c]Each value is an average of at least 3 tests.

lidaceae, were also assayed for their biocidal activity in comparison to products **2** and **3**. The compounds **5, 6,** and **7** were all prepared by synthesis from lycorine.

The results listed in Table 3 show that the antibiotic activity is correlated to the aromatization of the C ring present in the compounds **2, 3, 5,** and **6**. Furthermore, these data demonstrate that the different oxidation state of C-7 of **2, 3, 5,** and **6**, did not affect the antimicrobial activity.

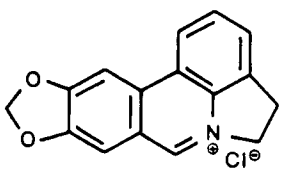
Compounds having a cyclohexenic C ring, such as lycorine and narciclasine (the latter also show radical structure modifications with respect to **1**) were devoid of activity. Conversion of **2** into its hydrochloride salt (**8**) caused a total loss of activity; the same result obtained when **7** was tested suggests that the ionization state of the phenolic hydroxy group, when it is present, probably is a feature essential to determine the activity.

Further studies are planned on the isolation of the enzymes responsible for the degradation of lycorine and on the biological implications of this transformation.



1

2



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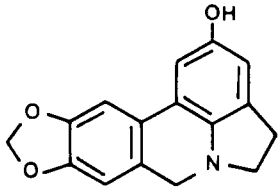
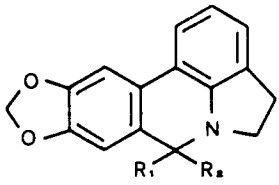
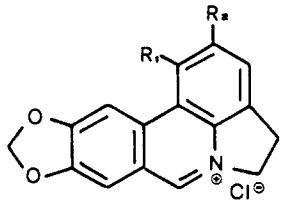


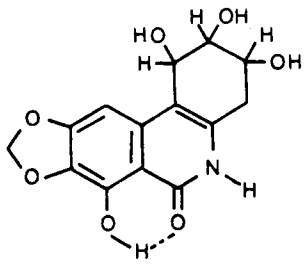
4



5 $R_1=R_2=H$
6 $R_1+R_2=O$



7 $R_1=OH$ $R_2=H$
8 $R_1=H$ $R_2=OH$



9

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points are uncorrected, ir spectra were recorded on a Perkin-Elmer 399 instrument, uv spectra were measured on a Varian Cary 210 spectrophotometer. ^1H- and ^{13}C-nmr spectra were recorded at 270 and 67.88 MHz, respectively, on a Bruker spectrometer; chemical shifts are in ppm. Mass spectra were recorded on AEI-30 mass spectrometer at 70 eV. Analytical tlc was performed on SiO_2 (Merck, Kieselgel 60 F_{254}, 0.25 mm) and on reversed phase ODS (Stratocrom C-18 Whatman 0.2 mm) prepared plates; the spots were made visible by exposure to I_2 or uv radiation. Column chromatography was carried out on Sephadex LH-20 (Pharmacia 25-100 μm).

SUBSTRATE.—Lycorine was obtained from bulbs of *S. lutea* according to our reported method (15).

PLANT MATERIAL.—*S. lutea*, collected near Bari, Italy, during the withering period, was identified by Prof. O. Arrigoni, Istituto di Botanica, Università di Bari, Italy (where a voucher specimen has been deposited).

BACTERIAL STRAIN.—Strain ITM 311 was isolated from rhizosphere of *S. lutea* plants by the methods described in Cuppels and Kelman (16). The strain was identified as being a species of *Pseudomonas* and deposited in the culture collection of the Istituto Tossine e Micotossine da Parassiti Vegetali del CNR, Bari, Italy. The culture media used in this study were ANG (nutrient broth, 0.8%; glycerol, 2%; agar, 1.5%) to maintain the bacterium, ANS (nutrient broth, 0.8%; sucrose, 5%; agar, 1.5%) to culture it, and minimal medium (MM) (K_2HPO_4, 0.3%; NaH_2PO_4, 0.1%; NH_4C, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.03%) supplemented with lycorine (MML) to isolate the bacterium (MML plus agar 1.5%) and evaluate its ability to degrade the lycorine (MML broth).

GROWTH OF STRAIN ITM 311.—Strain ITM 311 was grown on lycorine as the sole growth substrate in MM. The incubation was performed in 250-ml Erlenmeyer flasks containing 100 ml of broth, and the culture was incubated at 26° with agitation (250 rpm). The concentration of the growth substrate added to the medium was 0.2% (w/v) (7.0 mM). The inoculum was prepared from log-phase cultures of the strain on ANG slants. Cells were washed from slants, centrifuged (5000 g × 10 min), washed twice with saline (0.85%), and resuspended in MM to an optical density of 0.3 at 600 nm. Flasks were inoculated with 1 ml of this suspension. Growth was measured by the increase in viable counts, and the disappearance of the substrate was determined by tlc.

ANTIBACTERIAL ACTIVITY.—The antibacterial activity of the culture filtrate of strain ITM 311 grown on MML and of the products of its degradation were assayed in comparison to other related substances by the paper disc method. Bacterial cells of indicator strain were grown at 26° for 48 hr on ANG.

Cells were washed off the slants with about 4 ml of sterile H_2O, and 50 or 100 μl of the resulting suspension was mixed with 3 ml of molten 0.7% water agar. The inoculated water agar was poured over the surface of about 20 ml of ANS contained in Petri dishes. Paper discs with a diameter of 6 mm, impregnated with 20 μl of samples to be assayed, were deposited on the surface of the solidified aqueous agar layer. Plates were incubated for 48-72 hr at 26°, and the width of the inhibition zone was measured. In preliminary experiments, the antibacterial activity of the culture filtrate was assayed against *Pseudomonas cichorii* NCPPB2379, *Corynebacterium flaccumfaciens* NCPPB559, *C. fascians* NCPPB2551, *Pseudomonas syringae* pathovars *syringae* (NCPPB ITM24), *savastanoi* (ITM519), *tabaci* (NCPPB2730), *coronafaciens* (NCPPB2481), *tomato* (NCPPB2563) and *maculicola* (NCPPB1777), *Erwinia carotovora* subsp. *carotovora* NCPPB2577, *Agrobacterium tumefaciens* (ITM71), *Xanthomonas campestris* pv. *vesicatoria* (NCPPB 1616), *Bacillus megaterium* ITM80, and strain ITM 311.[1] All of the above were inhibited by culture filtrate except strain ITM 311, but as *C. fascians* was one of the most sensitive strains along with pv. *vesicatoria*, we chose to use the former bacterial species to assay the biocidal activity of the products of lycorine degradation (see Table 3).

PREPARATION OF BACTERIAL EXTRACT.—Washed suspensions were disrupted by sonication before centrifugation (20,000 g×20 min) and filter sterilization (Millipore, 0.45 μm). The resulting solution was used as a source of enzymatic extract. The protein content of the extract was determined by Lowry's method (18), using bovine serum albumin as the standard.

ISOLATION AND IDENTIFICATION OF THE PRODUCTS.—The culture filtrate (675 ml) was lyophilized, and the solid yellow-fluorescent residue (5.6 g) was extracted five times with MeOH (100 ml). The methanolic extracts analyzed by tlc on SiO_2 (*n*-BuOH-HOAc-H_2O, 60:15:25) and on reversed phase C-18 plates (H_2O-acetonitrile, 6:4) showed the presence of three products which fluoresced in different colors by exposure of the plates to uv radiation; moreover, the absence of lycorine was observed. Product (**2**) with a higher Rf (SiO_2 plates) was fluorescent rose, while the one with a lower Rf, i.e., the dihydroderivative of **2**, showed a brilliant sky-blue fluorescence. The product (**3**) with medium Rf fluoresced in a blue color.

Compound **2**.—The methanolic extracts of the culture filtrate were evaporated under reduced pressure to give a residue (848 mg) (still containing some salts present in the culture medium), which was then resuspended in H_2O (200 ml) and vigorously stirred in order to dissolve completely. The addition of 4 N NaOH to a pH value of 8-10, caused the precipitation of **2** in yellow flocculi. The mixture was decanted after keeping overnight at room temperature. The crude betaine was collected by centrifugation (10,000 g×45 min). Crystallization from EtOH yielded light orange needles of **2** (88 mg, 6.5%), which were collected by filtration. The product had no definite melting point and decomposed between 265 and 275°; uv λ max (0.1 N HCl and 0.1 N NaOH) nm (log ε) 258 (4.58), 3.61 (4.00) and 262 (4.57), 405 (3.90) respectively [lit. (11) decomp. between 260 and 270°; uv λ max (0.1 N HCl and 0.1 N NaOH) nm (log ε) 258 (4.7), 360 (4.1) and 262 (4.7), 408 (3.9)]; ir ν max (nujol) 1615 cm^{-1}; ^1H and ^{13}C nmr see Table 1 and 2, respectively; ms *m/z* (rel. int.) 265 (M^+) (67), 264 (100).

The hydrochloride of **2** (**8**).—Compound **8** precipitated as pale brown needles from an aqueous suspension of **2** by the addition of 10% HCl. The dried crystalline product did not melt below 350° [according to the literature (11)].

Compound **3**.—Compound **3** was obtained from the residue (97.6 mg) left by evaporation under reduced pressure of the ethanolic mother liquors derived from the crystallization of **2**. The purification of the mixture by repeated column chromatography (three times) on Sephadex LH-20 (eluent MeOH) produced **3** as an oily residue. The oily residue on treatment with MeOH and petroleum ether provided an amorphous solid (10.6 mg, 0.7%): decomp. 275-282°; uv λ max (EtOH) nm (log ε) 258 (4.34), 270 (4.36), 280 (4.33), 340 (3.87) [lit. (19): decomp. 280-285°; uv λ max nm (log ε) 258 (4.34), 270 (4.36), 280 (4.34), 341 (3.84)]; ^1H and ^{13}C nmr see Table 1 and 2, respectively; ms *m/z* (rel. int.) 250 (M^+) (51), 248 (100).

Compounds **5** and **6**.—Compounds **5** and **6** were prepared by treating lycorine (660 mg) with $POCl_3$ according to the described procedure (12). The crude residue obtained from the workup of the mixture reaction was fractionated by column chromatography on SiO_2 ($CHCl_3$-iPrOH, 95:5). The first substance eluted was compound **5** (31.4 mg, 4.7%) which crystallized from EtOH as pale brown prisms: mp 115-120°; uv λ max (EtOH) nm (log ε) 249 (4.32), 280 (3.85), 287 (3.82), 342 (3.87) [lit. (13): mp 122-123°; uv λ max (EtOH) nm (log ε) 217 (4.48), 249 (4.21), 281-289 (3.76), 342 (4.02), 352 (399), 357 (4.00)]; ir ν max ($CHCl_3$) 1630 cm^{-1}; ^1H nmr see Table 1; ms *m/z* (rel. int.) 251 (M^+) (50), 250 (100). The second compound eluted was **6**; the product was obtained as an amorphous solid by evaporation of the eluent under

[1]For the nomenclature of phytopathogenic bacteria we referred to the *Approved List of Bacterial Names* (17). NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, England; ITM, Collection of the Istituto Tossine e Micotossine da Parassiti Vegetali, Bari, Italy.

reduced pressure (14.1 mg, 2.1%): uv λ max (EtOH) nm (log ε) 242 (4.58), 246 (4.54), 273 (4.24), 309 (3.88), 324 (3.88), 341 (3.88) [lit. (13): uv λ max (EtOH) nm (log ε) 242 (4.66), 249 (4.62), 275 (4.32), 303 (4.03), 327 (3.84), 343 (3.86)]; ir ν max ($CHCl_3$) 1630, 1610 cm^{-1}; ^1H nmr see Table 1; ms *m/z* (rel. int.) 265 (M^+) (100), 264 (67).

Compound **7**.—Lycorine (1.0 g) was converted into **7** according to the reported procedure (11). The product (48.8 mg, 5%) crystallized from EtOH as yellow needles did not decompose by heating at 350°; uv λ max (0.1 N HCl and NaOH pH=10.0), nm (log ε) 257 (3.43), 295 (3.30) 410 (2.52) and 235 (5.37), 322 (5.20), 420 (4.70), respectively, [lit. (11): did not melt below 400°; uv λ max (0.1 N HCl and pH=10.0 buffer) nm (log ε) 257 (3.6), 295 (3.5), 407 (2.6) and 260 (5.5), 322 (5.6), 470 (4.4) respectively]; ^1H nmr was reported in Table 1; ms *m/z* (rel. int.) 265 (M^+), 264 (100).

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